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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 5 April 2002 with an application for Letters Patent number 518163 made by KIWI INGENUITY LIMITED.

Dated 23 April 2003.

Neville Harris

Neville Harris
Commissioner of Patents

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PROVISIONAL SPECIFICATION

MODIFIED EMBRYO

We, **KIWI INGENUITY LIMITED,** a New Zealand company of 47 Gosford Drive, Howick, Auckland, New Zealand do hereby declare this invention to be described in the following statement:

MODIFIED EMBRYO

This invention relates to an embryo which has been modified to enhance its implantation into the uterus. In particular, the invention relates to an embryo which has been modified by the insertion into the cell membrane (or zona pellucida) of a synthetically prepared molecule which has a binding affinity for endometrial cells.

BACKGROUND

Each year 15% of couples seek medical advice because of difficulties becoming pregnant (WHO 1997). Sub-fertility is therefore currently one of the most frequent health concerns facing the population aged 25-45. For the past two decades, *in vitro* fertilisation (IVF) has provided an effective form of assistance for a large proportion of these couples. Indeed, IVF now accounts for 1.3% of all live births Europe (Nygren *et al.* 2001) and 1.7% of all live births in Australasia (Hurst *et al.* 2001).

From the inception of routine IVF in 1978, pregnancy rates have risen steadily to levels considered normal for the fertile population (approximately 25% per attempt). The quest to break through this physiological barrier is driven by the significant financial and emotional cost for each IVF treatment for individuals.

Failure of embryos to implant into the lining of the uterus (endometrium) during an IVF treatment cycle is widely accepted by health professionals as the most significant limiting factor to improving success rates. There are two broad reasons for failure of implantation following replacement of apparently viable embryos. The first involves intrinsic embryonic factors that reflect retarded development or deficiencies in the health of the blastocyst itself and its ability to

hatch (Gott *et al* 1990, Plachot 1992, van Kooij *et al* 1996). The second relates to extrinsic factors that imply a lack of implantation receptivity in the endometrium (Edwards 1986, Yaron 1994). Moreover, successful implantation is dependent on the synchrony of embryonic development and endometrial maturation that is largely controlled by the ovarian hormone milieu.

Recently it has become apparent that fertility drugs used for the super-ovulation of women undergoing IVF are predominantly responsible for the compromised implantation receptivity observed on both sides of the embryonic/maternal interface. Ertzeid and Storeng cleverly demonstrated the detrimental effects of gonadotropins on implantation using a series of cross-over embryo transfer experiments (Ertzeid *et al.* 2001). Embryos from super-ovulated and non-stimulated females were transferred to separate uterine horns in the same super-ovulated or non-stimulated pseudo-pregnant recipient mice. A significant decrease in implantation was observed in the uterine horns receiving embryos from super-ovulated donors and even more dramatically in both horns of super-ovulated recipients.

Highly elevated concentrations of estrogen result from ovarian stimulation in IVF. These are suspected to alter the cascade of hormonal events and subsequent expression of cytokines that the oocytes, embryos and uterine endometrium would ordinarily be exposed to in an unstimulated menstrual cycle. Add to this the physiological challenge of *in vitro* culture, largely devoid of growth factors, and it is not unexpected that IVF derived embryos might be compromised at the time of implantation.

Despite substantial advances in the recovery and maturation of multiple oocytes from unstimulated cycles, the practice of oocyte *in vitro* maturation (IVM) is as yet clinically unaccepted. With the prospect that super-ovulation will remain the

mainstay of IVF, other approaches to improving implantation rates continue to be explored.

The development of physiological based culture media constituents has gone some way to improving the development of embryos in culture for up to 6 days. This extended culture enables self-selection of the most viable embryos for transfer, but as a consequence this approach has a high attrition rate of embryos. Co-culture of embryos on a mono- or bi-layer of support cells (e.g. endometrial cells) has also provided a method for improving the development of embryos in culture presumably via the stimulus of growth factors. More directly the addition of a variety of growth factors to media has been explored and shown to be of benefit (Sjoblom *et al.* 2000).

Maintaining a receptive endometrium through administration of human chorionic gonadotropin or progesterone has been practiced since the early days of IVF. In fact only after additional progesterone support was given in the luteal phase of the cycle, did the world's first IVF pregnancies result. It has long been recognised that the elevated estrogen profiles produced by the fertility drugs effectively advance the endometrial tissue dating by approximately one day (Noyes *et al.* 1950; Pittaway *et al.* 1983; Garcia *et al.* 1984). Compound this with the fact that embryos are routinely transferred into the uterus at the 2 – 8 cell stage (48-72 hrs prematurely to what occurs naturally) and it is clear that IVF results in an asynchronous environment for implantation.

Implantation of a hatched blastocyst is described as consisting of three phases:

a) apposition – where the embryo comes into initial physical contact with the glycoconjugate coat of the endometrial epithelium (called the glycocalyx).

b) adhesion – where the embryo undergoes cell to cell, and cell to matrix binding with molecules derived from the apical cells on the endometrium.

c) invasion – where the embryo penetrates through the epithelial layer of the endometrium by intruding between cell junctions as occurs in the human or by displacement of the cells found in some animals (e.g. mice).

Super-ovulation has been postulated to alter electronegative properties of the glycocalyx and apical cell surface of the endometrium. In this way, fertility drugs may reduce effective apposition and adhesion of a transferred embryo (Ronnberg *et al.* 1985).

At least two therapeutic approaches to improving implantation rates in IVF embryos have been practiced in humans. The first draws on the observation that inclusion of the glycosaminoglycan, hyaluronan, in the media containing embryos for transfer, results in a higher implantation rate than media devoid of this polysaccharide (Gardner *et al.* 1999). The concentration of hyaluronan increases in the uterus at the time of implantation in the mouse (Zorn *et al.* 1995) and is suggested to facilitate implantation by a variety of means such as increasing cell-cell and cell-matrix adhesion and indirectly through promotion of angiogenesis. Despite a lack of published trials in humans, hyaluronate is now present in a number of commercially available embryo transfer media.

One therapy that has undergone clinical trials and culminated in a patent specification, is the use of a fibrin sealant (Purdum 1999). The first experiments with a fibrin sealant were carried out in 1981, and by 1988 it had been proven safe to use in humans (Rodrigues *et al.* 1988).

US 6,196,965 is based on the technique used in a randomised clinical trial published in 1992 (Feichtinger *et al.* 1992). Embryos are transferred in a catheter, sandwiched between small quantities of thrombin/aprotinin and then fibrin. The results of the trial demonstrated no significant difference in pregnancy rate between the control and treatment group (546 patients), but a significant decrease in ectopic pregnancies in the fibrin sealant group.

The rationale and theoretical basis for the two therapeutic approaches described above are different. Hyaluronate is added to transfer media in the hope that it will induce a more physiologically receptive environment for implantation. There is, however, an absence of direct evidence at the molecular level proving this hypothesis. Fibrin sealant therapy on the other hand, is used to encase the embryos in an adhesive plug that will theoretically be glued onto the endometrium. Expulsion of embryos from the uterine cavity by muscular contraction and avoidance of ectopic pregnancy was the predominant motivation for the fibrin sealant in the Feichtinger trial (Feichtinger *et al.* 1990), although other investigators have hypothesised that fibrin would improve the adhesion phase of implanting embryos (Rodrigues *et al.* 1988).

In addition to the previously described therapeutic approaches, PCT/US98/15124 (published as WO 99/05255) describes the enhancement of implantation by contacting the embryo with a lipid-modified adhesion molecule. The technique of "protein painting" embryos with glycosylphosphatidylinositol (GPI) linked Qa-2 proteins to increase the cell division rate is described. While it is stated that the same methodology could be used to apply members of the adhesion family of proteins to the external membrane of embryos, there is no animal or human based evidence for this.

Protein painting is a method for modifying the external antigens of cell membranes without gene transfer. The method exploits the ability of GPI linked proteins to spontaneously anchor to cell membrane via their lipid tails. The method described in PCT/US98/15124 (WO 99/05255), requires that a naturally occurring (or genetically altered) protein, with an attached GPI lipid tail, is inserted into an embryo membrane with the expectation that the protein will maintain its known function. The molecules that can be used for modifying an embryo in this way are therefore confined to a rather limited group.

The inventors for this application have now found that embryos that have been modified with carefully selected chemically synthesised molecules have the ability to bind with endometrial cells. Not only has this been successfully demonstrated in an *in vitro* culture system, but animals have given birth to healthy offspring derived from modified embryos of this type.

It is therefore an object of this invention to provide an improved modified embryo for the enhanced implantation of the embryo into the endometrium of an animal, or to at least provide a useful choice.

STATEMENTS OF INVENTION

In a first aspect of the invention there is provided an embryo modified for enhancing the implantation of the embryo into the endometrium of an animal, where:

a) the embryo has an endometrial attachment molecule which is capable of attaching to the endometrium; and

b) the endometrial attachment molecule is linked to the embryo by a glycolipid having lipid tails inserted into the membrane of a cell of the embryo or into the zona pellucida of the embryo; and

c) the endometrial attachment molecule and the glycolipid have each been modified to incorporate a binding part enabling the endometrial attachment molecule and the glycolipid to bind together either directly or through a bridging molecule.

In one embodiment of the invention the endometrial attachment molecule and the glycolipid are bound by way of simple non-covalent binding interactions including ionic, van de Waals, water exclusion, electrostatic, hydrogen bonding and chelation binding. Alternatively, binding may be via covalent bonding.

In one embodiment of this invention the binding interaction between the endometrial attachment molecule and the glycolipid is avidin-biotin binding. In one preferred embodiment the binding part of the glycolipid is biotin and the binding part of the endometrial attachment molecule is avidin. In an alternative preferred embodiment the binding part of the glycolipid is avidin and the binding part of the endometrial attachment molecule is biotin.

In another embodiment of the invention the binding interaction between the endometrial attachment molecule and the glycolipid is through a bridging molecule. The bridging molecule may be avidin in the case of the binding part of both the endometrial attachment molecule and the glycolipid being biotin. Alternatively, in the case of the binding part of both the endometrial attachment molecule and the glycolipid being avidin, the bridging molecule may be biotin.

Alternatively, the binding interaction between the endometrial attachment molecule and the glycolipid may be a chelation interaction. The binding parts of

the endometrial attachment molecule and the glycolipid may therefore be bridged by a chelating metal (e.g. Co^{2+}, Ni^{2+} or Cu^{2+}) and a poly-histidine recombinant protein. The chelator may be attached covalently or non-covalently (via biotin or avidin) to the glycolipid.

The glycolipid may be any glycolipid capable of inserting its lipid tails into the membrane of a cell of the embryo or into the zona pellucida of the embryo. Preferably the glycolipid is of the ganglioside class that contains sialic acid groups.

The endometrial attachment molecule may be any molecule that has a binding affinity for molecules on endometrial cells (e.g. receptor sites and blood group related antigens). In particular, the endometrial attachment molecule is preferably a protein, a carbohydrate, or an immunoglobulin such as immunoglobulin G (IgG). Alternatively, the endometrial attachment molecule may be a synthetic molecule (e.g. polyvinyl pyrrolidine) which reacts with molecules expressed on endometrial cells or on the mucus layer covering the endometrium.

In a second aspect of the invention there is provided a method of preparing the modified embryo of the first aspect of the invention including the steps:

a) contacting an endometrial attachment molecule with a glycolipid, where the endometrial attachment molecule and the glycolipid have each been modified to incorporate a binding part enabling the endometrial attachment molecule and the glycolipid to bind together either directly or through a bridging molecule; and then

b) contacting the endometrial attachment molecule bound to the glycolipid with an embryo so that the lipid tails of the glycolipid insert

into the membrane of a cell of the embryo or into the zona pellucida of the embryo;

or including the steps:

c) contacting a glycolipid with an embryo, where the glycolipid has been modified to incorporate a binding part capable of binding to an endometrial attachment molecule either directly or through a bridging molecule, so that the lipid tails of the glycolipid insert into the membrane of a cell of the embryo or into the zona pellucida of the embryo; and then

d) contacting the glycolipid inserted into the embryo with an endometrial attachment molecule, where the endometrial attachment molecule has been modified to incorporate a binding part capable of binding to the glycolipid either directly or through a bridging molecule.

In a first embodiment of the invention the method of preparing the modified embryo involves steps a) and b). In a second embodiment the method involves steps c) and d).

Preferably the glycolipid has been modified to incorporate biotin as its binding part and the endometrial attachment molecule has been modified to incorporate avidin as its binding part.

Alternatively, the glycolipid has been modified to incorporate avidin as its binding part and the endometrial attachment molecule has been modified to incorporate biotin in its binding part.

In the case of binding of the glycolipid to the endometrial attachment molecule through a bridging molecule, it is preferred that the bridging molecule is avidin

and that both the glycolipid and the endometrial attachment molecule have been modified to incorporate biotin as their respective binding parts.

In another aspect of the invention there is provided the implantation of an embryo of the first aspect of this invention into the endometrium of an animal, preferably a human.

In a further aspect of the invention there is provided a method of enhancing the implantation of an embryo into the endometrium of an animal, preferably a human, comprising the steps:

a) preparing a modified embryo according to the second aspect of this invention, and

b) transferring the modified embryo to the uterus of the animal.

DETAILED DESCRIPTION

Terms or expressions used to describe this invention are defined as follows:

i. endometrium - the tissue lining the internal surface of the uterus. It is this layer of epithelial cells and its extracellular matrix (i.e. mucus) that the implanting embryo comes into first contact with. The epithelial and underlying stromal cell layer cyclically thickens, secretes mucus and is shed from the body under the hormonal influence of the menstrual cycle.

ii. zona pellucida - the glycoprotein coat that surrounds the mammalian oocyte (egg) and embryo from the 1-cell to blastocyst (6 day old) stage of development. Prior to embryo attachment in implantation, the zona pellucida is shed from the embryo by a number of

mechanisms including proteolytic degradation. The zona pellucida functions initially to prevent entry into the oocyte by more than one sperm, then later to prevent premature adhesion of the embryo before its arrival into the uterus.

iii. endometrial attachment molecule - any protein, glycoconjugate or synthetic molecule that can react with a protein or glycoconjugate or related molecule present on the endometrial cell surface or mucus layer.

iv. glycolipid - any lipid-containing carbohydrate, including phosphoglycerides (e.g. glycosylphosphatidylinositol) and sphingolipids (e.g. glycosyl ceramides, cerebroside sulfate, and gangliosides).

v. binding part (of endometrial attachment molecule or of glycolipid) - the portion of the molecule that interacts (or docks) with the endometrial attachment molecule or glycolipid, or with a bridging molecule.

vi. bridging molecule - a molecule that links the glycolipid with the endometrial attachment molecule, for example, avidin (interacting with biotin on either the glycolipid or the attachment molecule), and a chelator (interacting with a poly-histidine).

vii. biotin - biotin is vitamin H. It consists of fused imidazolinone and thiophan rings with a pentanoate side-chain attached to the latter. Biotin has an extremely high affinity for binding with the protein avidin via its imidazolidine ring.

viii. avidin (Av) - avidin is a glycoprotein with a molecular mass of 67 kDa when derived from chicken egg white. Avidin contains four identical sub-units, each bearing a biotin-binding site.

ix. chelation - chelation is defined as the strong binding that occurs between chelated metal ions and proteins. Certain chemical groups called ligands, such as iminodiacetate and nitrilotriacetate, form a stable metal coordination complex (or metal chelate) with a divalent transition metal ion, e.g. Ni^{2+}, Co^{2+} or Cu^{2+}. Peptides containing poly-histidine residues strongly bind to such a metal chelate by participation of imidazole side-chains in chelation.

x. BioG (Biotinylated ganglioside) - biotin coupled to a ganglioside.

xi. BioIgG (Biotinylated Immunoglobulin G) - biotin coupled to immunoglobulin G.

The insertion technology used for this invention is based on the established principle that glycolipids can insert into cell membranes without damaging cells. This principle underpins the inventors' development which is the insertion of synthetically prepared molecules into the glycoprotein coat of early embryos (zona pellucida) and the lipid bi-layer membrane of embryonic and endometrial cells that are involved in embryo implantation.

While this technology is applicable to embryo implantation in a wide variety of animals, it is most relevant to humans. However, this invention is not limited to human embryo modification and implantation.

One or several intercellular interactions can be targeted for improvement using the technology of this invention. This may be a direct adhesion mechanism, or other mitotic stimulus or cell recognition events. While the endometrial attachment molecule and glycolipid may be derived from natural or synthetic sources, the assembly of the endometrial attachment molecule and the glycolipid is synthetic. Furthermore, the covalent or non-covalent attachment of the endometrial attachment molecule may occur either before or after the insertion of the glycolipid into the cell membrane.

The following description of this invention relates to biotin/avidin binding. But it is important to note that any combination of endometrial attachment molecule and glycolipid (each having a binding part) which allows high affinity conjugation (i.e. covalent or non-covalent bonding) is suitable.

One combination that employs biotin/avidin binding is a biotinylated glycolipid as the primary insertion molecule, an avidin bridging molecule, and a biotinylated endometrial adhesion molecule (for example, an antibody). The insertion process operates by exploiting the high binding affinity of avidin for biotinylated molecules, essentially forming a sandwich complex. Firstly, the biotinylated glycolipid is inserted into the cell membrane to provide an anchor for the application of subsequent molecules. Secondly, the inserted cell membrane is treated with avidin that binds to the biotinylated glycolipid. The final phase involves conjugation of the inserted molecules with the biotinylated endometrial adhesion molecule. To demonstrate this invention, the endometrial adhesion molecule is the immunoglobulin G antibody. However, it must be emphasised that this molecule could be substituted by any one of a variety of biotinylated molecules.

Immunoglobulin G was chosen for development of the invention because of the ease in which molecular insertion and cell adhesion between two cell types can be confirmed using serological techniques. Preliminary development for each phase of the invention was carried out using human RBCs and the addition of antiserum resulted in an agglutination reaction (see Example 7). Thereafter, the insertion technique was tested on mouse embryos ranging from the 2-Cell to blastocyst stage of development. The successful insertion of the endometrial adhesion molecule in embryonic membranes was tested by the addition of anti-IgG and IgG-sensitised human RBCs representing surrogate endometrial cells.

At each developmental phase, it was important to investigate the potential risk of detrimental effects of the invention on embryonic development and maternal health. Initially, the morphological development of treated embryos was compared with control embryos cultured in vitro. The outcome of normal live births from transferred treated embryos into recipient mice provides evidence of the safety of the invention. Finally, the ongoing reproductive performance of the treated offspring proves that no lasting detrimental effects are present.

Insertion of Endometrial Attachment Molecules into Embryos

A solvent free glycolipid insertion media was developed (see Example 10) to ensure protection from the reported detrimental effects of alcohols in sensitive embryonic cells (Lau *et al.* 1991). The extraction and purification of porcine gangliosides was carried out using established techniques (Karlsson 1987, Ladisch *et al.* 1987, Ledeen *et al.* 1982). Synthesis of the biotinylated ganglioside was performed using the technique outlined in Example 8.1. The insertion solution is an aqueous medium. The results in Example 10, are in agreement with other investigators that the presence of serum, plasma or detergents is unnecessary for insertion to occur (Zhang *et al.* 1992). In contrast

with previous reports, the presence of albumin in the M2 media in Example 10, does not impede the insertion process. Therefore, the insertion solution is effective in culture media with and without the presence of protein. Example 1 clearly demonstrates successful insertion of the primary insertion BioG molecule into zona intact and zona free embryos ranging from the 2-Cell to blastocyst stage.

Adhesive Properties of Endometrial Attachment Molecule Inserted Embryos

Human IgG-sensitised RBCs were used essentially as surrogate endometrial cells to demonstrate the adhesive properties of embryos inserted with BioIgG as the endometrial attachment molecule. Example 2 uses a serological technique known as Rosetting (Indiveri *et al* 1979), where the addition of antiserum to inserted embryos causes adhesion of IgG-sensitised red blood cells. In this way it was possible to prove that not only had the BioIgG terminal molecule been successfully inserted into the embryo, but that an artificial adhesion between two cell types had been created.

Viability of Endometrial Attachment Molecule Inserted Embryos

An essential requirement of any implantation therapy is that it must not induce any detrimental effects on the normal fetal growth of the treated embryo or future reproductive fitness of the offspring and mother. Preliminary experiments with BioG inserted embryos showed no difference in morphology or zona hatching rate from control embryos during 5 days of *in vitro* culture (Example 3). Similarly, no difference from control embryos was noted (although not subjected to statistical analysis) between the pregnancy, live birth rate and normalcy of offspring in treated embryos (BioG, BioG/Av/BioIgG, ZI and ZF) when transferred into recipient mice (Example 4 and Example 5). Ultimately, the ongoing fertility

rate and second generation pups of the offspring resulting from treated embryos was apparently normal (Example 6).

EXAMPLES

Example 1

Insertion of biotinylated gangliosides (BioG) into the cell membranes of both zona pellucida intact (ZI) and zona pellucida free (ZF) murine embryos from 2-Cell stage through to hatched blastocyst stage was confirmed by a positive signal of avidin conjugated to fluoresceinisothiocyanate (avidin-FITC) detected under fluorescent microscopy. Some ZI embryos underwent zona removal post BioG insertion and pre avidin-FITC treatment to clearly visualise the degree of BioG insertion in the cell membrane. Embryo insertion was performed in both M2 (Sigma M5910) and SQC (Vitrolife, Sweden) media using the following method:

1. Collection of super-ovulated mouse embryos on Day 1.5 to Day 3.5 post coitus was performed as described in Example 4.

2. Embryos from each mouse were split equally between control and experimental groups where possible and transported from the animal facility to laboratory in separate sterile microcentrifuge tubes with M2 media.

3. A 4-well culture dish was prepared with 4x 50 µl micro-drops of media overlaid with mineral oil and the following reagents in separate drops: a) 5 µl of BioG (50 mg/ml stock), and b) 5 µl of avidin-FITC (1 mg/ml). The dish was equilibrated in a 5% CO_2, 37 °C incubator for 90 minutes when

SQC media (bicarbonate buffered – not suitable for atmospheric conditions) was used or 30 minutes at 37 °C when M2 media (HEPES buffered for atmospheric conditions) was used.

4. Embryos destined for ZF insertion treatment were placed in 0.5% protease (Sigma P8811) in M2 media for 6 minutes at 37 °C until the zona had disappeared.

5. All embryos were washed 3 times in M2 media after each treatment step by placing them into a fresh 100 μl drop of media using a pulled glass capillary tube and syringe.

6. ZF and ZI embryos were placed in the BioG micro-drop for 1-2 hours under appropriate culture conditions.

7. A group of ZI embryos were treated with 0.5% protease prior to further treatment.

8. Embryos were subsequently cultured in the avidin-FITC drop for 1 hr in dark culture conditions.

9. Embryos were mounted on a glass microscope slide in a 2 μl drop of Citiflour (R1321, Agar Scientific, NZ) and overlaid with 2 μl of mineral oil, to replace the need for a cover-slip. A felt tip marker was used to circle the location of the specimen.

10. The slides were viewed under a fluorescent microscope at 250-500x magnification using a 470 nm filter.

The results of each experiments performed are outlined in Table 1.

Table 1. Fluorescent signal observed in mouse embryos inserted with BioG

Expt	Embryonic stage	Outline	Result
1	4 cell-cultured from 2 cell	Four groups of 4 cells a) ZF controls b) ZF experimental c) ZI controls d) ZI experimental	Signal: a) Nil b) + + + c) Nil d) + + +
2	unhatched and hatched blastocysts cultured from 2 cell	Four groups of 5 embryos a) Hatched controls b) Hatched experimental c) Unhatched controls d) Unhatched experimental e) arrested embryos All treated with BioG then Avidin-FITC (24hrs later)	Signal: a) Nil except for atretic cells b) + + (stronger in atretic cells) c) nil except for atretic cells d) + + + to + + + + zona e) + + + cell contents, nil on zona No difference in morphology between control and experimental 24hrs post BioG exposure
3	Late morula cultured from 2 cell	Three groups of 8 embryos a) Control embryos b) BioG then pronase c) Pronase then BioG	Signal: a) Faint homogenous signal b) + + to + + + cell outline c) + + + to + + + + cell outline
4	Blastocyst to hatched blastocyst cultured from 2 cell	Two groups of 5 embryos a) BioG and Avidin-FITC Day 6 b) BioG Day 2 with further culture then Avidin-FITC	Signal: a) + + to + + + + zona, very faint internal b) + + to + + + zona and faint internal signal from hatched blasts
5	Hatching blastocyst	Four groups of 3 embryos BioG treatment 24 hours previous a) Pronase then Avidin-FITC b) Avidin-FITC then Pronase c) No Pronase old Avidin – FITC d) No Pronase new Avidin -	Signal: a) + + + to + + + + cell outline b) + + + to + + + + cell outline

		FITC	c) + + + to + + + + zona and fainter internal cell outline d) + + + + zona and fainter internal cell outline
6	2 cell embryos freshly retrieved	Four groups of 5 embryos a) M2 media controls b) M2 media experimental c) SQC media controls d) SQC media experimental	Signal: a) Nil b) + + + + zona, clear cell outline c) Nil d) + + + + zona, clear cell outline

Example 2

The ability of modified embryos to adhere specifically (through a series of immunological bridges) to antigens on other cell types was tested. In this example, the adhesion molecule was classified as biotinylated IgG and anti IgG [BioIgG + anti IgG]. This adhesion molecule was conjugated in a third step to the inserted molecules BioG and avidin on the cell membranes and zona pellucida of murine embryos. To confirm the complete insertion of this complex, IgG sensitised RBCs were allowed to adhere to the embryos in a serology technique known as Rosetting. This technique is used for identifying cells by mixing them with particles or cells to which they bind (Indiveri *et al* 1979). The rosettes consist of a central cell surrounded by bound cells. The IgG that is attached to the sensitised RBCs is used as an antigen for the adhesion molecule. Therefore these cells essentially act here as surrogate endometrial cells.

Insertion, conjugation and adhesion of IgG sensitised RBCs was carried out as follows:

1. Sensitised RBCs were made by incubating 400 µl of human serum containing human anti IgG with 200 µl of RhD + ve human RBCs for 1 hr at

37 °C. The RBCs were then washed in Celpresol and made up to a 5% solution for the rosette technique.

2. All embryos were retrieved from super-ovulated mice at the 2-Cell stage and entered into the experiment either on the day of retrieval or after 48 hours of cell culture in SQC media (late morula to blastocysts stage).

3. BioG insertion was performed on both zona intact and zona free embryos as described in Example 1 with either M2 or SQC used as the insertion media.

4. The embryos then underwent a 3rd conjugation step where they were exposed to 5 µl of avidin (1 mg/ml) in a 50 µl of micro-drop of media for 90 minutes at 37 °C in appropriate culture conditions for each media type (i.e. CO_2 or atmospheric).

5. The washed embryos underwent a second conjugation with 5 µl of BioIgG (1mg/ml) in a micro-drop of media for 30 minutes at 37 °C, then washed.

6. The embryos were placed in a micro-drop consisting of 25 µl of M2 media and 25 µl of monoclonal anti IgG for 30 minutes at 22 °C.

7. The treated and control embryos were washed and placed in separate drops of M2 media. A stream of either 5% IgG sensitised RBCs or 5% untreated RBCs were gently blown over the embryos using a pulled capillary pipette attached to a mouth-piece.

8. After 10 minutes at room temperature, the embryos were gently transferred to fresh media micro-drops using a wide bore capillary pipette

(170 μm diameter) and assessed for RBC adherence under an inverted microscope at 250x magnification.

The results are shown in Table 2, Table 3 and Table 4.

Table 2. The degree of adhesion observed for BioG inserted and Av/BioIgG + anti IgG conjugated 2-Cell zona intact embryos exposed to either IgG-sensitised or untreated RBCs

Experimental Group	No. embryos	Adherence
Negative control M2 insertion media Embryos exposed to untreated RBCs	5	-
M2 insertion media Embryos exposed to sensitisd RBCs	5	+ + +
Negative control SQC insertion media Embryos exposed to untreated RBCs	5	-
SQC insertion media Embryos exposed to sensitised RBCs	5	+ + +

Table 3. The degree of adhesion for BioG/Av/BioIgG + anti IgG inserted Day 5 stage cultured embryos (late morula to blastocyst) exposed to either IgG-sensitized or untreated RBCs

Experimental Group	No. embryos	Adherence
Treated embryos Exposed to sensitised RBC	5	+ + +

Negative control Treated embryos Exposed to untreated RBC	5	-
Negative control Untreated embryos Exposed to sensitised RBC	5	-
Negative control Untreated embryos Exposed to untreated RBC	5	-

Table 4. The degree of adhesion for BioG/Av/BioIgG + anti IgG Day 6 stage cultured zona intact and zona free embryos (blastocyst to hatched blastocyst) exposed to either IgG-sensitised or untreated RBCs

Experimental Group	No. embryos	Adherence
Treated zona intact embryos Exposed to sensitised RBCs	3	+ + +
Negative control Treated zona intact embryos exposed to untreated RBC	3	-
Treated zona free embryos Exposed to sensitised RBCs	3	+ +
Negative control Treated zona free embryos exposed to untreated RBC	3	-

The adhesion of large quantities of sensitised RBCs to embryos (2-Cell to blastocysts) indicates positive insertion of Bio/Av/BioIgG and demonstrates the ability of transformed embryos to adhere. There was no difference in the adhesion score between M2 and SQC insertion media. The adhesion score was moderately greater in the zona intact embryos than the zona free embryos.

Example 3

The viability of murine embryos following BioG insertion treatment was confirmed by continued culture and assessment of morphological development. Eleven 2-Cell mouse embryos underwent BioG insertion (as described in Example 1) with subsequent wash steps and culture in a 50 µl micro-drop of SQC media overlaid with mineral oil. Sixteen control embryos were cultured in a separate micro-drop in the same 4-well culture dish (Nunc 176740). Forty-eight hours later there was no difference in morphology between the experimental and control embryos. All embryos had reached the expected late morula to early blastocyst stage of development. Equal numbers of embryos initiated zona hatching by Day 5 of culture.

Example 4

The viability of murine embryos treated with biotinylated ganglioside (BioG) was confirmed by the presence of implantation sites and live birth of pups after embryo transfer (ET) into recipient mice. The retrieval, treatment, and transfer of embryos were carried out on the same day at the animal facility. All embryo manipulations, molecular insertions and incubations were performed in M2 HEPES buffered media on a 37°C heated microscope stage.

<u>Donor Superovulation and Embryo Retrieval</u>

Large and relatively predictable numbers of embryos can be collected for experiments by using fertility drugs to stimulate the ovaries of immature mice which are highly sensitised to follicle stimulating hormone (FSH).

Prepubescent (<35 day old) CBA/C57 F1 female mice were injected with 5IU of FSH (Folligon, Pharmaco, NZ) at 1700 and again 48 hours later with 5IU of human chorionic gonadotrophin (Pregnyl, Organon, NZ). Each mouse was immediately placed with a CBA male stud mouse of proven fertility and checked for a seminal plug the following morning. The donors were sacrificed by cervical dislocation on the morning of either Day 1.5 post coitus for the retrieval of 2-Cell embryos or Day 3.5 for late morula to blastocysts. The uterine horns were excised from the abdomen using sterile technique and placed into a plastic petri dish where they were flushed with media to expel the embryos.

Embryo BioG Insertion

An equal number of high quality embryos were selected from each donor flushing and pooled together for experimental and control groups. Experimental embryos were placed in a 50 µl micro-drop of M2 media that had been conditioned with 2.5-5 µl of BioG (50 mg/ml) for 1-1.5 hrs at 37°C. The embryos were washed three times with M2 and placed in a micro-drop of M2 in preparation for transfer. Control embryos were processed through drops of media only at the same time as experimental embryos.

Embryo Transfer (ET)

To obtain a receptive endometrium in recipient mice, it is necessary to create a state of pseudopregnancy by mating with a vasectomised male mouse. The act of coitus rescues the corpus luteum of ovulated follicles from demise and sustains progesterone production necessary for implantation to occur.

Recipient CBA/C57 F1 female mice in estrus (40-120 days old), were selected from the pool of mice and placed with a vasectomised male mouse of proven sterility. The time of mating was programmed so that recipients were

synchronous for 2-Cell embryos transfers or asynchronous by minus 1 day for blastocyst stage transfers. Only recipients exhibiting a clearly identified seminal plug the following morning were selected as recipients.

The recipient mice were anaesthetised with 0.8 ml of Avertin (made in-house) and an incision was made in the side of the abdomen above the hip. The fat pad above the ovary was grasped with a serrafin clamp to withdraw the oviduct and uterus outside of the body. Using a 23-28 gauge needle, a hole was made in either the bursa of the ovary to expose the infundibulum for 2-Cell stage transfers, or the uterine horn for blastocyst stage transfers. Six to ten embryos were loaded (using a mouth piece) into a fire pulled and polished capillary pipette (approx. 150-170 μm in diameter) with mineral oil and air gaps to stabilise the embryos. The pipette was inserted into the prepared needle puncture site and the embryos expelled until the release of an air-gap was visible. The exposed reproductive tract was replaced into the abdominal cavity and the body wall and skin closed with suture. The mouse was identified with ear marking and observed until conscious.

Mice were house singularly in cages until they were either sacrificed for identification of implantation sites or until they had given birth. The implantation (imps) and live birth (pups) rates are presented in Table 5.

All recipients were kept for 3-6 months post exposure to BioG for health assessment. The offspring were maintained for breeding of one litter to assess reproductive fitness in the second generation.

Table 5. Implantation and live birth outcomes of BioG treated 2-Cell and blastocyst (blast) stage embryos

Exp	Treatment	No. embryos transferred	Preg Y/N	No. imps. or pups	Comment
1	Control	6x 2-Cell	Y	Pups x 5 83%	Born 19 days post ET – normal healthy pups
2	Control	6x 2-Cell	N	-	-
3	BioG + Control	6x 2-Cell BioG 6x 2-Cell Control	Y	Imps. 2 BioG 2 control 33%	Each group separated into separate uterine horns Sacrificed Day 7 pregnancy
4	BioG + Control Different coloured babies	5x 2-Cell BioG black mice 5x 2-Cell control grey mice	Y	Pups x5 (2x grey and 3x black)	Same uterine horn ET No difference in morphology or growth between BioG and control mice ET mum died 1 day before weaning – ?stress
5	BioG + Control Different coloured babies	5x 2-Cell BioG black mice 5x 2-Cell control grey mice	Y	Pups x5 (1x grey and 4x black)	Same uterine horn ET No difference in morphology or growth between BioG and control mice
6	BioG	10 x 2-Cell	N	-	Died 3 months post ET
7	BioG	10 x 2-Cell	Y	Pups x 8 80%	Born 18 days post ET Normal healthy pups
8	BioG	10x 2-Cell	Y	Pups x8 80%	Normal healthy pups
9	BioG	7x 2-Cell	Y	Pups x6 85%	Normal healthy pups
10	Control	8 x 2-Cell	Y	Pups x5 63%	Born 18 days post ET Normal healthy pups
11	Control	10 x 2-Cell	Y	Pups x6 60%	Born 20 days post ET Normal healthy pups (1x runt)
12	BioG	6x blasts	Y	Imps x4 66%	Uterine ET Sacrificed D10 of pregnancy Day 2.5 recipient

Exp	Treatment	No. embryos Transferred	Preg Y/N	No. imps. Or pups	Comment
13	Control	6x blasts	Y	Imps x6 100%	Uterine ET Sacrificed D10 of pregnancy Day 2.5 recipient
14	BioG	6x blasts	Y	Imps x4 66%	Uterine ET Sacrificed D10 of pregnancy Day 2.5 recipient
15	Control	8x blasts	Y	Imps x5 63%	Uterine ET Sacrificed D10 of pregnancy Day 2.5 recipient
16	Control	6x blasts	Y	Pups x4 66%	Day 3.5 Recipients Normal healthy pups
17	BioG	6x blasts	Y	Pups x3 50%	Day 3.5 Recipients Normal healthy pups
18	BioG	6x blasts	Y	Pups x5 83%	Day 3.5 Recipients Normal healthy pups

The first indication that BioG 2-Cell embryos were capable of implantation was in a recipient mouse that had 6 BioG inserted embryos replaced into one uterine horn and 6 untreated control embryos replaced into the other horn. An inspection of the excised uteri on Day 7 of pregnancy revealed 4 implantation sites in each horn.

The second experimental evidence showed that not only were BioG 2-Cell embryos capable of implantation but they also gave rise to live healthy pups. In experiments 4 and 5, five embryos derived from a pure black strain of mice (C57 donor and stud) were inserted with BioG and replaced into the same uterine horn as five control embryos derived from a pure grey strain of mouse (CBA donor and stud). The resulting colour of the 10 offspring, combined from both recipient mothers, was 3 grey (control embryos) and 7 black (BioG) babies.

Further ET experiments utilising embryos at two different stages of development, 2-Cell and blastocyst, revealed similar pregnancy and live birth rates between BioG embryos and untreated control embryos for both stages of development. Overall, 8 out of 9 embryo transfers of BioG embryos only resulted in a pregnancy with a live birth rate of 72.0%. The transfer of control embryos resulted in a pregnancy for 6 out of 7 ETs, with a 72.5% live birth rate.

In conclusion, the insertion of BioG in zona intact embryos from 2-Cell to blastocyst stage does not appear to significantly impair the implantation and ongoing development of the embryo to live birth of healthy pups.

Example 5

The viability of zona free and zona intact murine embryos, inserted with biotinylated ganglioside (BioG) and conjugated sequentially with avidin (Av) and biotinylated IgG was confirmed by the birth of live pups post embryo transfer. Embryo retrieval and transfer of blastocysts was carried out using the methodology previously described in Example 4. Embryo insertion and conjugation was performed as described in Example 2.

Table 6. Pregnancy outcome and live births of BioG/Av/BioIgG embryos zona intact and zona free.

Exp.	Treatment type	No. embryos transferred	Preg. Y/N	No. imps or pups	Comment
19	BioG/Av/BioIgG	6x blasts zona intact	Y	Pups x 5 83%	Born 16 days post ET Normal healthy pups
20	Control	6x blasts zona intact	N	-	-
21	Control	6x blasts zona intact	Y	Pups x2 33%	Born 16 days post ET Normal healthy pups
22	BioG/Av/BioIgG	6x late morula zona intact	Y	Pups x3 50%	Born 17 days post ET Normal healthy pups
23	BioG/Av/BioIgG	6x late morula zona intact	Y	Pups x3 50%	Born 17 days post ET Normal healthy pups (1x died)
24	Pronase BioG/Av/BioIgG	6x blasts zona free	Y	Pups x6 100%	Born 16 days post ET Normal healthy pups (1x died)
25	Pronase BioG/Av/BioIgG	6x blasts zona free	Y	Pups x6 100%	Born 16 days post ET Normal healthy pups
26	Pronase Control	6x blasts zona free	Y	Pups X5 83%	Born 17 days post ET Normal healthy pups
27	Pronase Control	6x blasts zona free	Y	Pups x4 66%	Born 16 days post ET Normal healthy pups
28	Pronase BioG/Av/BioIgG	6x blasts zona free	Y	Pups x3 50%	Born 16 days post ET Normal healthy pups

The data outlined in Table 6 showed similar live birth rates were observed for experimental and control treated embryos in both the ZI and ZF groups (experimental and control respectively: ZI 61% vs 33%, ZF 83% vs 71.5%). The primary aim of this series of experiments was not to compare implantation or live birth rates, hence the small numbers and subsequent lack of statistical analysis is irrelevant. The results do however confirm that ZI and ZF embryos inserted with the complete BioG/Av/BioIgG molecule give rise to healthy live pups.

Example 6

The reproductive fitness of experimental offspring proved to be similar to other inbred mice within the same animal facility. Offspring from embryo transfer experiments were paired in cages and allowed to breed. All pairs produced a litter within 75 days of birth. The mean size of the litter was 6.2 pups with normal appearance (Table 7).

Table 7. Number of pups delivered in 1st litter from experimental offspring.

Experimental Origin and Pair	Treatment	No. of pups in 1st litter
7	BioG	8
8	BioG	5
9	BioG	8
19	BioG/Av/BioIgG	4
22	BioG/Av/BioIgG	7
21	Control ZI	5

23	BioG/Av/BiolgG	10
24	Pronase BioG/Av/BiolgG	6
25	Pronase BioG/Av/BiolgG	3

General Methods

Example 7

The following example is one method for testing the presence of inserted antigens on RBCs:

1. Washed inserted RBCs were made to a 5% concentration by adding Celpresol or saline.

2. 25 µl of the RBC suspension was added to a small glass tube. 25 µl of antiserum was added.
3. The RBC suspension and the antiserum were mixed well and spun in an immunofuge for 15 seconds.

The degree of agglutination was assessed and scored as follows:

Table 8. Agglutination scoring method

Agglutination Score	Observations
-	No clumps at all
(+)	Indeterminant
+	Very small clumps
++	Several small clumps
+++	On large clump surrounded by small clumps
++++	On single large clump

The higher the concentration of antigen glycolipid solution used for insertion, the greater the amount of antigen inserted into the RBCs.

Example 8.1

Biotinylated gangliosides (BioG) were prepared using a modified procedure described by Wilchek and Bayer (1987)

1. Dried gangliosides purified from porcine brains, were reconstituted in PBS with the aid of sonication.

2. The ganglioside sialic residues were oxidized by the addition of sodium m-periodate.

3. The solution was subjected to 24 hr dialysis to remove the resulting peroxide.

4. The oxidised ganglioside was incubated with biotin amidocaproyl hydrazide (Sigma B-3770). for 1hr.

5. The solution was subjected to further overnight dialysis in water to remove excess biotin amidocaproyl hydrazide

6. The resulting solution was dried via rota evaporation and reconstituted in 50% methanol water. Further evaporation was performed under nitrogen gas in a reduced pressure desiccator overnight.

Example 8.2

Biotinylated immunoglobulin G was prepared using a method described by O'Shannessy (O'Shannessy 1990). Using similar procedures to those outlined in Example 8.1, the IgG was oxidised with a periodate solution and incubated with biotin amidocaproyl hydrazide.

Example 9

A solvent free glycolipid insertion media was developed specifically to protect sensitive cells such as embryos from solvent exposure during insertion treatment.

1. Purified dried glycolipids (either Le^b, Grp A, or biotinylated ganglioside) were dissolved in a glass tube with 50% methanol/water to give a 10 mg/ml solution.

2. The solution was filtered with a 0.22 micron solvent resistant filter into a sterile glass tube.

3. A 150 µl aliquot of the solution was marked on the side of the glass tube to indicate the end point of evaporation. A further 850 µl aliquot was placed in the glass tube.

4. The tube was placed under a gentle stream of nitrogen gas in a dry heat block at 50°C until the meniscus was reduced to the marked evaporation line (approx 30 minutes).

5. The solution was made up to 200 μl with a balanced salt solution of sterile PBS by adding 20 μl of 10x phosphate buffered saline (PBS) and 30 μl of 18 mΩ water.

6. The final 50 mg/ml solution was aliquoted into sterile microcentrifuge tubes and frozen at −70°C or freeze dried.

Example 10

The requirement for plasma or serum in the insertion media was shown not to be necessary. The ability for fluorescent-labelled avidin- (avidin-FITC Sigma A-2901) to bind to biotin formed the basis of detecting inserted BioG in RBCs when viewed under microscope fluorescence at 470 nm. In this study, a comparison in the degree of fluorescent signal in avidin-FITC treated BioG human RBCs was carried out for insertion solutions containing the following media:

1. Celpresol (CSL, Biosciences, Australia)

2. M2 mouse embryo handling media (Sigma M5910) containing HEPES buffer and 10% bovine serum albumin (BSA)

3. SQC mouse culture media (Vitrolife, Sweden), protein free

4. Medicult human embryo culture media (Medicult, Denmark) containing 10% synthetic serum substitute.

5. PBS (in-house made).

The concentrations and fluorescent microscopy results are outlined in Table 9.

Table 9. Fluorescent signal of Avidin-FITC indicating degree of BioG insertion in RBC membranes when performed in plasma and plasma free media.

	Plasma-free media				Plasma media	
Insertion Media	Celpresol	M2	SQC	PBS	Serum	Plasma
Concentrations	5ul BioG 50mg/ml in 25µl Celpresol +5µl RBC	5ul BioG 50mg/ml in 25µl M2 +5µl RBC	5ul BioG 50mg/ml in 25µl SQC +5µl RBC	5ul BioG 50mg/ml in 25µl PBS + 5µl RBC	5ul BioG 50mg/ml in 25µl serum + 5µl RBC	5ul BioG 50mg/ml in 25µl Plasma + 5µl RBC
Experimental 5µl inserted RBC + 9.5µl avidin (1mg/ml)	+++	++++	+++	++	++	++
Control 5µl untreated RBC + 9.5µl avidin (1mg/ml)	-	-	-	-	-	-

S = Fluorescent signal 0, +, ++, +++, ++++ (least to greatest)

The presence of a clear fluorescent signal in both M2 and SQC media deemed them to appropriate for routine embryo insertion experiments.

Example 11

Optimum BioG insertion concentrations and conditions were established by labelling the inserted BioG RBCs with avidin-FITC as described in Example 10. The results are outlined in Table 10.

Table 10. Fluorescent signal emitted from human RBC inserted with decreasing concentrations of BioG and Avidin-FITC.

	Bio G (50mg/ml) 5μl .25mg + 25μL SQC media + 50μl RBC	Bio G (50mg/ml) 2.5μl .125mg + 27.5μl SQC media + 50μl RBC	Bio G (50mg/ml) 1.20μl .06mg + 28.8μl SQC media + 50μl RBC	Control 30ul SQC media + 50μl RBC
Avidin-FITC (1mg/ml)	5μl Packed RBC and resuspend in 30μL media SQC			
30μl (30μg)	+++	++++	+++	-
15μl (15μg)	++++	++++	+++	-
10μl (10μg)	++++	++++	+++	-
5 μl (5μg)	++++	++++	+++	-

S = Signal 0, +, ++, +++, ++++ (least to greatest)

The optimum insertion concentration of BioG was 10 mg/ml (2.5 μl of BioG 50 mg/ml in a 25 μl drop of media). The minimum concentration of Avidin required for adequate detection of BioG at 10 mg/ml concentration, was 0.16 g/ml (5 μl of Avidin-FITC 1 mg/ml in a 30 μl drop of media). The optimum time for insertion was determined to be 1 hour as seen in Table 11.

Table 11. Maximum BioG insertion time for human RBCs as determined by the fluorescent signal of Avidin-FITC

Tube	Insertion time	<u>Bio-G (50mg/ml)</u> 2.5μL (.125mg) + 27.5μL SQC media + 50μL packed RBC (5μL Avidin –FITC + 5ul RBC + 30ul SQC media)
1	15 min	+++
2	30 min	+++
3	45 min	+++
4	1 hr	++++
5	1.5hr	++++
6	2 hr	++++
8	4 hr	++++
10	6 hr	++++
12	26 hr	++++

The amount of fluorescent signal score for tube 6 reduced from 4+ to 3+ after Avidin labelling 5 days post insertion, suggesting minimal loss of inserted molecules over time.

Example 12

Human RBCs were inserted with BioG followed by subsequent conjugation, with Avidin (Sigma A9275, un-labelled) then BioIgG. Successful insertion and conjugation was determined by serology testing (described in Example 7) with a range of antibodies to IgG.

Insertion, conjugation and agglutination of RBCs was carried out as follows:

1. 1 ml of packed group O RBCs was washed 3x with 1.3% saline in a microfuge tube for 1minute at 300 g and made up to a 5% solution with Celpresol

2. 1 µl of BioG was added to 1 ml of the 5% solution of washed RBCs and incubated on a gentle shaker for 1.5 hours at 35°C

3. The samples were washed 3x and resuspended up to a 5% solution with Celpresol

4. 20 µl of avidin (1 mg/ml) solution was added to 180 µl of the 5% inserted RBC solution and incubated on a shaker for 1 hour at 35°C

5. The samples were washed 3x with Celpresol and resuspended up to 200 µl.

6. BioIgG was added to 50 µl of 5% inserted RBC solutions at the concentrations outlined in Table 12. The samples were incubated for 37°C for 30 minutes then washed 3x with Celpresol.

7. Serology testing was carried out and the results are tabulated in Table 12.

Table 12. Agglutination scores for BioG/Av inserted RBCs with varying concentrations of BioIgGs using polyclonal and monoclonal anti IgG

	BioIgG 1mg/ml	BioIgG 0.5mg/ml	BioIgG 0.25mg/ml	BioIgG 0mg/ml
BioG/Av inserted RBCs	A ++++ B ++++	A ++++ B ++++	A ++++ B ++++	A - B <1+
Negative Control Untreated RBCs	A - B -	A - B -	A - B -	A - B -
Negative Control BioG inserted RBCs with no Av	A-	A-	A-	A-
Positive Control IgG sensitised RBCs	A +++ B +++			

A. anti polyclonal IgG/monoclonal C3d - mouse
B. anti IgG monoclonal – mouse

A strong positive agglutination reaction seen in all BioG/Av/BioIgG treated RBCs after the addition of both monoclonal and polyclonal Anti IgG, confirms that successful insertion and conjugation had occurred.

Example 13

The minimum concentration of BioIgG required to elicit a positive serology agglutination test in BioG/Av/BioIgG inserted human RBCs was determined using the methodology outlined in Example 7. A negative control consisted of RBCs inserted with a solution of ganglioside only (1 µl of 50 mg/ml ganglioside in 1000 µl of 5% RBCs. The test solution consisted of a 5% solution of RBCs in Celpresol inserted with biotinylated ganglioside (5µl of 50 mg/ml Bio G in 1000 ul of 5% RBC) and conjugated with 10 µl of Avidin (1 mg/ml). The results are shown in Table 13.

Table 13. Serial dilutions of BioIgG used for the final conjugation step for the insertion of biotinylated ganglioside and conjugation of Avidin in human RBCs

	1:50 Bio IgG 4 µl Bio IgG + 196 µl media (50µl of this solution + 50µl RBC (5%)	1:100	1:200	1:400	1:800	1:1600	1:3200	Zero 200µl of Celpresol only
Negative Control Gang RBCs	-	-	-	-	-	not done	not done	-
BioG RBCs	++++	++++	++++	+	++	-	-	-

Serial dilutions of BioIgG conjugation solutions revealed an acceptable agglutination reaction at 1:200 upon addition of polyclonal Anti IgG/C3d.

Although the invention has been described by way of example, it should be appreciated that variations and modifications may be made without departing from the scope of the invention. Furthermore, where known equivalents exist to specific features, such equivalents are incorporated as if specifically referred in this specification.

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